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Enzyme-Linked Immunosorbent Assay for *Pseudomonas aeruginosa* Exotoxin A

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An enzyme-linked immunosorbent assay (ELISA) is described for *Pseudomonas aeruginosa* exotoxin A. A double antibody sandwich method was used, employing polyvinyl microtiter plates as the solid phase, a primary coat of monospecific rabbit antitoxin serum, an outer layer composed of a horseradish peroxidase–sheep antitoxin immunoglobulin G conjugate, and an ortho-phenylene-diamine substrate. Absorbance (optical density) of hydrolyzed end product was read spectrophotometrically at 492 nm. ELISA detected as little as 30 pg (0.3 ng/ml) of purified toxin, and absorbance was linear over a 20-fold or greater concentration range. Toxin was demonstrated in culture filtrates from 42 of 48 (88%) consecutive clinical *P. aeruginosa* isolates compared with 37 of 48 (77%) positive by hemagglutination inhibition. Results of the two assays correlated closely ($r = 0.82$, $P < 0.001$). Specificity was confirmed by neutralizability of ELISA activity with monospecific antitoxin. ELISA was thus a sensitive, specific, and quantifiable technique for the assay of *P. aeruginosa* exotoxin A in both purified and crude culture materials.

Exotoxin A (8) is a protein synthesis-inhibiting extracellular enzyme (5) that is produced by the majority of clinical *Pseudomonas aeruginosa* strains of all immunotypes (16). Purification of the toxin (3, 7, 11, 17) and preparation of monospecific antisera (4) have facilitated the development of biological (6, 9, 10, 14, 16), immunological (1, 2, 10, 15, 16), and enzymatic assays (2, 5) for both toxin and antitoxin. We describe here an enzyme-linked immunosorbent assay (ELISA) for toxin which is sensitive, specific, and quantifiable.

MATERIALS AND METHODS

Organisms and culture preparation. Forty-eight consecutive *P. aeruginosa* isolates were obtained from the Bacteriology Laboratory, National Naval Medical Center, Bethesda, Md. The following *P. aeruginosa* strains were also used: PA 103, a toxigenic (tox^+), low protease-producing ($prot^-$) Fisher-Devlin type 2 sputum isolate (8); PA 86, a tox^+, $prot^+$, type 3 blood isolate (13); and WR5, a tox^-, $prot^+$, type 6 blood isolate (2, 13). A *P. cepacia* strain (ATCC 25416) was obtained from the American Type Culture Collection, Rockville, Md. Filtrates were prepared from overnight cultures grown in Trypticase soy broth dialysate with 5 mM nitrilotriacetic acid, 5 mM monosodium glutamate, and 1% (vol/vol) glycerol (16).

Exotoxin A and antitoxin. Toxin was purified from PA 103 cultures by affinity chromatography using a Sepharose 4B column containing covalently bound sheep antitoxin immunoglobulin G (17). Toxin batch no. 11577 used in this study migrated as a single band on 10% sodium dodecyl sulfate-polyacrylamide gels containing 30 μg of protein, yielded a single precipitin line by immunodioffusion with either antitoxin serum or antiserum to crude PA 103 culture filtrate, and had a median lethal dose for 20-g NIH-NMRI CV strain female Swiss mice of 75 ng.

Monospecific rabbit and sheep antitoxin sera were prepared as previously described (14, 17). An immunoglobulin G fraction used for making the enzyme conjugate was prepared from sheep antitoxin serum by ammonium sulfate precipitation and diethylaminoethyl ion-exchange chromatography (17).

ELISA. Polyvinyl microtitration plates (Cooke Laboratory Products, Alexandria, Va.) were coated with 100 μl of rabbit antitoxin serum diluted in 0.01 M carbonate buffer (pH 9.6) and incubated overnight at room temperature. The plates were then rinsed five times with working buffer (WB), composed of 0.036 M borate-buffered saline (pH 7.8) containing 0.5% bovine serum albumin fraction V (Calbiochem, La Jolla, Calif.) and 0.5% Tween 20 (Sigma Chemical Co., St. Louis, Mo.). The last rinse was kept in the wells for 15 to 30 min. The test specimens were diluted in WB and added to the coated wells in 100-μl volumes. After 2 h of incubation at 37°C, the wells were rinsed five times, 100 μl of horseradish peroxidase–sheep antitoxin immunoglobulin G conjugate (12) diluted in WB was added, and the plates were incubated again at 37°C for 2 h. After rinsing five more times with WB and once with substrate buffer, 100 μl of substrate (0.04%

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ortho-phenylene-diamine [Eastman-Kodak, Rochester, N. Y.] plus 0.003% H_2O_2 in 0.05 M phosphate-citrate buffer, pH 5.8) was added, and the plates were incubated in the dark at 37°C for 60 min. Reactions were stopped by the addition of 140 μl of 1.3 N H_2SO_4 to each well, and optical density of the end products was read at 492 nm on a spectrophotometer fitted with a rapid sampling device and ultramicrocuvette (Stasar II, Gilford Instrument Laboratories, Inc., Oberlin, Ohio). All samples were run in triplicate, and optical density readings were averaged. Background controls consisted of horseradish peroxidase-sheep antitoxin conjugate plus substrate in WB or in culture media diluted in WB. Mean optical density readings ≥ twice background were considered positive (i.e., indicative of the presence of toxin). For screening purposes, culture filtrates were run at 1:8 dilution in WB.

Neutralizations were accomplished by the incubation of equal 180-μl samples of purified toxin or culture filtrates and rabbit antitoxin serum, diluted 1:200, at 37°C for 1 h in polystyrene microtiter plates (Limbro Chemical Co., Inc., New Haven, Conn.) prior to transfer of the toxin-containing samples to ELISA plates.

HI assay. Chromium chloride-sensitized, exotoxin A-coated sheep erythrocytes were used to measure toxin in culture filtrates by hemagglutination inhibition (HI) assay (17). End points were determined using twofold dilutions of culture material. Titers were expressed as $\log_2$ of their reciprocals, and for statistical purposes titers of <2 were considered to be 1.

RESULTS

Optimal dilutions of rabbit antitoxin used to coat microtiter wells and of horseradish peroxidase-sheep antitoxin conjugate (1:1,000 and 1:200, respectively) were chosen on the basis of dose-response experiments performed at various toxin concentrations. Lower dilutions of antitoxin "coat" failed to increase sensitivity, whereas larger amounts of conjugate increased background in rough proportion to test sample activity, thereby nullifying any potential gain in sensitivity.

Titrations by ELISA of purified toxin and toxin contained in culture filtrates are shown in Fig. 1. The lowest detectable concentration of purified toxin (mean optical density of triplicate samples ≥ twice background) was between 0.3 and 0.6 ng/ml, or as little as 30 pg per microtiter well. The relationship between optical density and doubling dilutions of toxin was linear over

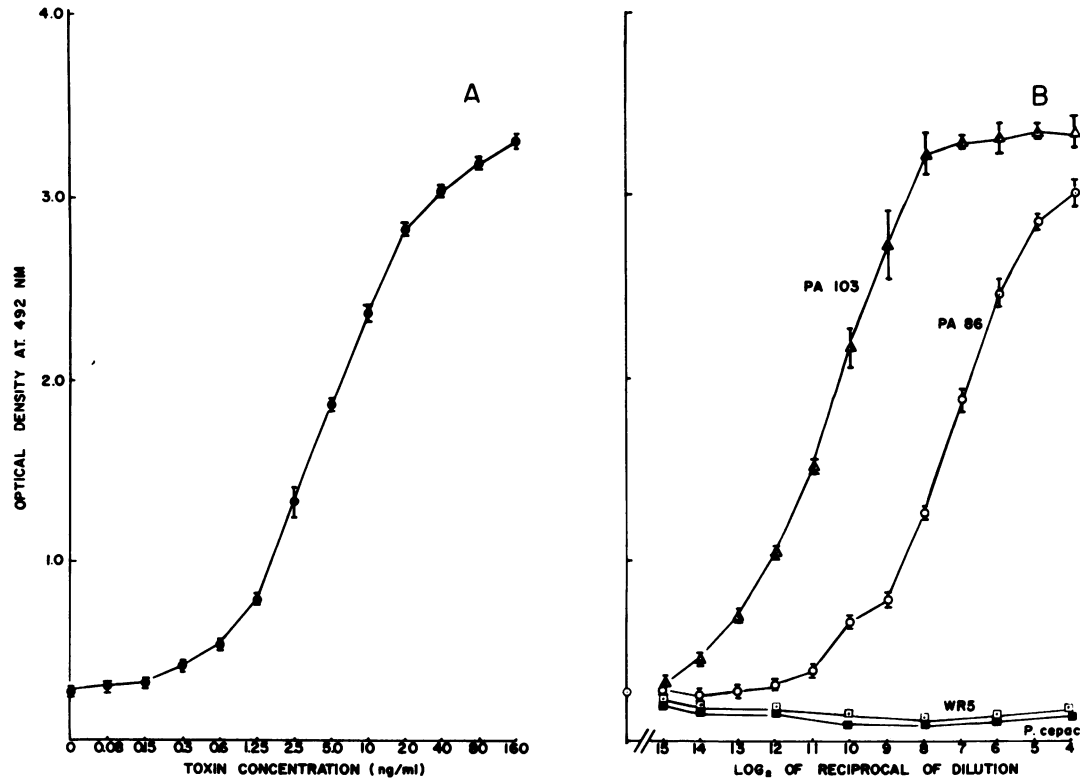

FIG. 1. *ELISA of P. aeruginosa exotoxin A. Relation of absorbance of end product to (A) concentration of purified toxin and (B) dilution of culture filtrates. P. aeruginosa strains PA 103 (△) and PA 86 (○) were tox⁺, and WR5 (□) was tox⁻. A P. cepacia strain (■) was also included. Data points and brackets indicate the means of triplicate tests ± standard error of the mean.*

a concentration range of 1.25 to 20 ng/ml (Fig. 1A). Similarly, there was a linear relationship between optical density and twofold dilutions of *P. aeruginosa* culture filtrates over a 16- to 32-fold dilution range (Fig. 1B). In contrast, the absorbance of culture filtrates of the tox$^-$ WR5 and *P. cepacia* strains was essentially that of background, even at lower dilutions (Fig. 1B).

The specificity of ELISA is demonstrated by the neutralization data in Fig. 2. Incubation of purified toxin or culture filtrate from a tox$^+$ strain with monospecific antitoxin serum prior to assay resulted in complete loss of measurable absorbance above background levels at antitoxin dilutions as high as 1:2,000. The absorption of antitoxin serum with whole heat-killed organisms prior to its use in neutralizing toxin contained in culture filtrates derived from the same strain insured the specificity of the neutralization. (This procedure did not reduce the antitoxin titer of the serum.) The absence of neutralization after incubation of toxin (Fig. 2) or culture filtrates with normal rabbit serum or antitoxin serum previously absorbed with purified toxin (data not shown) provided further evidence of specificity.

The correlation ($r = 0.82$, $P < 0.001$) between HI titers and absorbance measured by ELISA of 48 *P. aeruginosa* cultures is shown in Fig. 3. The greater sensitivity of ELISA compared with HI is demonstrated by the fact that, of 48 cultures, 37 were positive for toxin by both assays, 5 negative by HI were positive by ELISA, and none negative by ELISA was positive by HI. Since all positive ELISA results were confirmed by neutralization using highly specific antitoxin, the five negative HI results appear to be due to insufficient sensitivity. The apparent difference in sensitivity of the two assays was probably underestimated, since the lowest culture dilution used for ELISA was 1:8 and that used in HI was 1:4. Repeated titrations of purified toxin by ELISA demonstrated roughly 10-fold greater sensitivity compared with HI (data not shown).

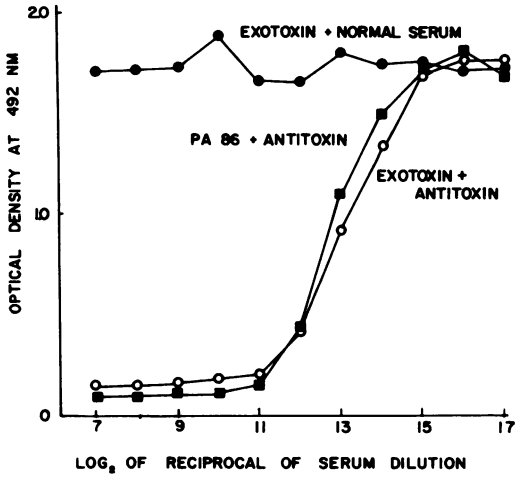

FIG. 2. *Neutralization of P. aeruginosa exotoxin A, as measured by ELISA. Constant amounts of purified toxin (final concentration = 2.2 μg/0.1 ml) were preincubated with monospecific rabbit antitoxin (○) or normal rabbit serum (●), in the dilutions shown, prior to ELISA. In addition, crude culture filtrate from tox$^+$ P. aeruginosa strain PA 86 diluted 1:100 was preincubated with antitoxin (■) in the dilutions shown, prior to assay by ELISA for unneutralized toxin.*

DISCUSSION

We have described an ELISA for *P. aeruginosa* exotoxin. Our ELISA results correlated closely with HI toxin titers of 48 *P. aeruginosa* culture filtrates, and ELISA was the more sensitive test. Since absorbance of ELISA end products is a continuous function of toxin concentration, over a rather wide range, the assay is quantitative and the need for multiple sample dilutions is eliminated. As shown, ELISA is useful for quantifying purified toxin as well as toxin contained in crude culture material. The appli-

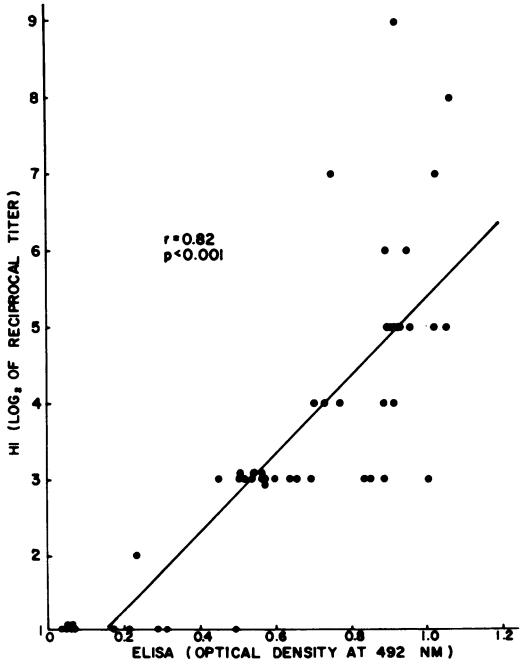

FIG. 3. *Assay of exotoxin A in culture filtrates from 48 consecutive P. aeruginosa clinical isolates: correlation of HI titers and absorbance measured by ELISA. Regression line was computed by the method of least squares, and the P value was determined from the statistic* $t = r\sqrt{n-2}/\sqrt{1-r^2}$.

cability of the assay to the demonstration of toxin in clinical material is under study.

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